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SPORIDIAL FUSION IN USTILAGO MAYDIS [1]

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INTRODUCTION

Cytological and cultural investigations of *Ustilago maydis* (DC.) Cda.[3] reported in the literature have left certain phases of the knowledge concerning sporidial fusion and nuclear phenomena in this species in a state of controversy. Maire (*10*) [4] in 1898 suggested that fusion of sporidia occurred in culture. He showed one illustration of fusion but did not describe the nuclear condition. Later Sartoris (*14*) reported the occurrence of sporidial fusion in culture but did not describe or illustrate the process. Seyfert (*15*) was unable to confirm the work of Sartoris. Rawitscher (*11*) reported that the sporidia neither fused nor formed true mycelium but remained uninucleate. Hanna (*4, p. 430*) studied the physiology and cytology of sporidia in *U. zeae* and *Sorosporium reilianum* (Kuehn) McAlp. and concluded that ". . . it seems clear that the conditions which stimulate this process in many other smuts are without effect on the sporidia of *U. zeae*." Christensen (*2*) found it necessary to inoculate the host plant with paired cultures in order to study segregation of sex factors. Sleumer (*16*) obtained sporidial fusion in culture between compatible sporidia in *U. zeae*. His descriptions and illustrations of the process agree essentially with those given for other members of the Ustilaginaceae.

In view of the conflicting and incomplete information contained in the literature, the work reported herein was undertaken to determine (1) conditions conducive to sporidial fusion in culture and (2) the nuclear behavior following fusion.

MATERIAL AND METHODS

The chlamydospore material of *Ustilago maydis* used in this investigation was collected from inbred lines of corn (*Zea mays* L.) grown at Madison, Wis. Sporidial cultures of monobasidiospore (monosporidial) origin were used and were established by taking single basidio-

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[2] Grateful acknowledgment is due Dr. J. G. Dickson for helpful suggestions and criticisms throughout the course of this investigation.

[3] Its synonym, *Ustilago zeae* (Beckm.) Unger, is used in many articles cited.

[4] Italic numbers in parentheses refer to Literature Cited, p. 242.

spores from the basidium (promycelium) by the method described by Hanna (*3*).

Stock cultures were maintained on potato-dextrose agar. In the sporidial studies 1-percent plain, refined agar medium or dilute malt-extract liquid medium was used. A malt-extract stock solution was prepared from freshly ground malt used at the rate of 10 gm. per 100 cc. of distilled water. The extraction was carried out at temperatures of 40°, 50°, and 65° C. for successive periods of 10, 20, and 30 minutes, respectively. The liquid was then decanted and autoclaved. A subsequent filtration to remove the precipitate occasioned by autoclaving was necessary if a clear liquid was desired. This stock solution was used at the rate of 1 part to 100 parts of distilled water, unless otherwise stated.

Cultures of paired monosporidial lines were prepared for study on ordinary glass slides or as hanging-drop cultures on deep-well slides. Sporidia from 3- to 4-day-old potato-dextrose agar slants were used throughout these studies.

RESULTS

PHYSIOLOGY OF SPORIDIAL PRODUCTION AND FUSION

Sporidial fusion in certain of the Ustilaginaceae has been reported to be influenced by the nutrient concentration and reaction of the culture media (*5*, *6*, *14*). Sleumer (*16*) observed that sporidial fusion in *Ustilago zeae* occurred on 3-percent maltose or glucose media only after the nutrients were spent. However, he reported fusion also on malt-extract medium after 24 hours. He believed that fusion was most likely to occur when the reaction of the medium was pH 8.0 to 8.5. Kernkamp (*7*) found three growth types in *U. zeae:* (1) A sporidial type, (2) a mycelial type, and (3) different intergrades of an intermediate type. The growth type used in the present studies obviously was of the intermediate type in that it produced both sporidia and mycelium. Several preliminary tests were conducted with a mixture of compatible monosporidial cultures that were known to cause gall formation when inoculated into corn plants. These tests indicated that (1) the development of haploid hyphae by the sporidia and (2) the fusion of compatible sporidia occurred only in cultures of low nutritive value. Only vegetative budding of the sporidia was observed in cultures with nutrient concentrations common in many laboratory media. These findings are in agreement with those of Kernkamp (*7*), who concluded that increasing the quantity of various nutrients, especially certain sugars, increased the sporidial growth in cultures of the intermediate type. He reported also that some cultures of the intermediate type were predominantly sporidial in nature when grown in solutions of relatively high nutritive concentration. However, as the cultures aged and the supply of nutrients decreased, the cultures became more mycelial in nature.

Additional tests were made with the same monobasidiospore lines in hanging-drop cultures in sterile distilled water and in malt extracts of 1 and 2 percent. After sterilization, the reaction of distilled water ranged from pH 7.0 to 7.2 and that of the malt extracts from pH 6.8 to 7.0. These cultures were incubated at various temperatures, of 4° intervals, from 4° to 36° C. and were examined after 24, 48, and 72 hours. Each test was made in duplicate and repeated once. The average results of the tests are given in table 1.

At the end of 24 hours, haploid hyphae and fused sporidia were evident in the distilled-water cultures incubated at temperatures of 20° C. or above. Sporidial production and growth rather than fusion appeared to be stimulated slightly in the 1-percent malt-extract cultures. At the end of 48 hours, in the 2-percent malt-extract cultures, the fewest haploid hyphae occurred and there were no sporidial fusions at any temperature. At the end of 72 hours, the percentages of haploid hyphae and sporidial fusions in the 2-percent malt-extract cultures had increased slightly, except at the lowest temperatures, but the percentages were still the lowest. In the cultures in all three media sporidial fusions were found to increase with advancing age. At two temperatures, approximately 6 percent of the sporidia in the distilled-water cultures had fused at the end of 72 hours.

TABLE 1.—*Influence of time, temperature, and culture media on the development of sporidia, haploid hyphae, and fusion pairs in Ustilago maydis*

Time (hours)	Temperature	Distilled water				1-percent malt extract				2-percent malt extract			
		Sporidia		Haploid hyphae [2]	Fusion pairs [2]	Sporidia		Haploid hyphae [2]	Fusion pairs [2]	Sporidia		Haploid hyphae [2]	Fusion pairs [2]
		Production [1]	Length [1]			Production [1 2]	Length [1]			Production [1]	Length [1]		
	° C.			Percent	Percent			Percent	Percent			Percent	Percent
24	36	2	2	25	T	2	2	10	0	3	3	0	0
	32	2	2	25	T	2	2	10–12	T	3	3	0	0
	28	2	2	20	T–1	2	2	10–12	T	3	2	0	0
	24	1	2	15	T–1	2	2	10	T	3	2	0	0
	20	1	1	10	T	2	2	5	0	3	2	0	0
	16	0	1	5	0	1	1	1–2	0	2	2	0	0
	12	0	1	0	0	1	1	0	0	1	1	0	0
	8	0	1	0	0	T	1	0	0	1	1	0	0
	4	0	1	0	0	T	1	0	0	1	1	0	0
48	36	2	3	40	----	3	4	20	----	4	4	3	0
	32	2	3	40	----	3	3	20	2–5	4	4	3	0
	28	2	2	30	3	3	3	20	2–5	4	3	2	0
	24	2	2	25	3–4	2	2	15	2	4	3	1–2	0
	20	1	1	15	3	2	2	10	1	3	2	T	0
	16	1	1	5	T–1	2	2	5	0	3	2	0	0
	12	1	1	----	0	1	1	T	0	2	1	0	0
	8	1	1	0	0	1	1	0	0	1	1	0	0
	4	1	1	0	0	1	1	0	0	1	1	0	0
72	36	2	3	40	----	3	4	30	----	4	4	5	T
	32	2	3	40	----	3	3	30	3	4	4	5	T
	28	2	2	30	4–6	3	3	30	3	4	4	3	T
	24	2	2	30	6	3	2	25	3	4	3	3	1
	20	2	2	25	3–4	2	2	20	1	3	3	1	T
	16	1	1	10	T	2	2	10	T	3	2	T	0
	12	1	1	T	0	1	1	T	0	2	1	0	0
	8	1	1	T	0	1	1	0	0	1	1	0	0
	4	1	1	0	0	1	1	0	0	1	1	0	0

[1] 0=No increase; 4=maximum increase; based on mean values.
[2] T=Trace.

The temperatures at which the cultures were incubated also influenced the incidence of sporidial fusion. Sporidia produced at the higher temperatures, 28°, 32°, and 36° C., were long and narrow. This condition, together with the rapid multiplication of sporidia by vegetative budding at these temperatures, made the identification of haploid hyphae and fusion pairs difficult and uncertain. In general, a decrease in temperature was accompanied by decreases in the number and size of sporidia, in the number and length of haploid hyphae,

and in the prevalence of fused sporidia. No sporidial fusion and only a small amount of growth occurred in the cultures held at 12° or lower for 72 hours. These cultures and those incubated at the lower temperatures were continued for an additional 7-day period. At that time a few fused sporidia were observed both in the distilled-water and in the 1-percent malt-extract cultures incubated at 12°. No fusion was observed at the lower temperatures.

For a closer study of sporidial fusion, cultures were prepared from paired sporidial lines of monobasidiospore origin in sterile distilled water and in 1-percent malt-extract solutions. These cultures were incubated at 20° and 24° C. Fusion was observed first after 15 and 20 hours in the malt-extract cultures incubated at 24° and 20°, respectively, and after 20 hours in the distilled-water cultures incubated at 24°. Fusion pairs were not abundant, however, until the cultures were 40 to 48 hours old. This confirms the work of Sleumer (*16*) with

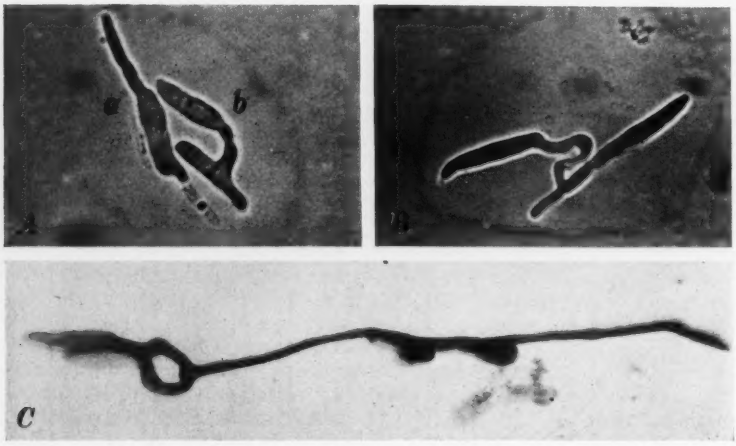

FIGURE 1.—Sporidial fusion in *Ustilago maydis*. Approximately ×1,200. *A*, *a*, Sporidium germinating, with uninucleate hyphae developing from both ends of the spore; *b*, an early stage of fusion between two compatible sporidia, with the binucleate hypha developing from the end cell of one sporidium. *B*, An early stage of fusion between sporidia, similar to that in *A*, *b*. *C*, A later stage in sporidial fusion, in which the fusion hypha is several cells long. *A* and *B* were stained with acid fuchsin and *C* with Delafield's haematoxylin.

the same fungus in respect to time required for fusion to take place. The fusion process between compatible sporidia of *Ustilago maydis* differs only slightly from that reported for certain other smuts. The conjugation process did not seem to follow any definite pattern such as that described by Holton (*6*) for *U. avenae* and *U. levis*. Neither sporidium appeared to take a more active part than the other. Fusion appeared to fall into three general types: (1) Direct fusion of two adjacent sporidia; (2) fusion of young one- or two-celled hyphae (germ tubes) formed from sporidia close to each other; and (3) fusion of cells of two older uninucleate mycelia. The uninucleate hyphae by means of which the fusions occurred were found to vary considerably in diameter and length. Representative stages of fusion are

shown in figure 1. In figure 1, *A*, *a*, uninucleate hyphae have developed from both cells of a two-celled, nonfused sporidium. Early stages of fusion are illustrated in figure 1, *A*, *b*, and *B*. In each case the fusion hypha has just started its development. A somewhat later stage in which the fusion hypha is several cells in length is shown in figure 1, *C*. In this case the sporidia have not fused directly although in juxtaposition, but rather fusion has taken place between the short hyphae developed from each.

Although fusion was more commonly observed between compatible sporidia close to each other, it was observed also between cells of uninucleate hyphae originating from widely separated sporidia. This type of fusion is not peculiar to *Ustilago maydis*, as a similar type of fusion has been reported by Stakman, Cassell, and Moore (*17*) for *Urocystis occulta* (Wallr.) Rab. In a few instances compatible sporidia that had apparently fused end to end by means of short germ tubes were observed. In such cases the binucleate hypha developed from the opposite end of one sporidium.

The hyphal outgrowth initiating the binucleate condition develops soon after the fusion of two compatible gametes. The hyphae grow rapidly and under favorable conditions often reach a considerable length. Occasionally it was possible to follow binucleate hyphae that had grown across the culture drop for distances of approximately 5 to 8 mm. (5,000μ to 8,000μ). Sleumer (*16*) observed one such hypha that had reached a length of 340μ.

Holton (*6*) and Harper (*5*) reported that the hyphae developed from fused sporidia in *Ustilago avenae* and *U. levis* and in *U. antherarum*, respectively, revert to the production of sporidia eventually. Sleumer (*16*) stated that the addition of nutrient material to cultures of *U. zeae* resulted in sporidial formation by both uninucleate and binucleate mycelia. The possible influence of adding nutrients to the cultures was not investigated by the writers. Hanging-drop cultures were maintained in the laboratory for 2 months, however, without any evidence of sporidial formation from binucleate hyphae. Frequently uninucleate hyphae were found to produce aerial sporidia at the surface of the culture drop. The binucleate hyphae varied considerably in size and often were no coarser than the uninucleate hyphae. Hence, it became necessary to trace any hypha in question to its origin or resort to staining procedure to determine its nuclear condition.

NUCLEAR BEHAVIOR AFTER FUSION

When sporidial cultures had reached the desired stage for staining, they were killed with Flemming's weaker solution or Carnoy's alcohol-acetic acid solution or simply by drying them rapidly over a low flame. When thoroughly dried, the cultures were found to adhere to the slide or cover slip sufficiently well to permit their manipulation in the staining procedure without appreciable loss.

The protoplasmic contents in the rapidly growing portions of both uninucleate and binucleate hyphae exhibited a marked affinity for certain dyes and destained very irregularly. This was especially true when crystal violet was used. Either Heidenhain's or Delafield's haematoxylin gave greater uniformity. The latter stain was more satisfactory because the cell walls of the preparations thus stained were more readily discernible. A weak concentration of acid fuchsin in

lactophenol was found to be fairly satisfactory as a general, semipermanent, rapid stain.

The procedure followed with Heidenhain's iron alum haematoxylin was essentially the same as that described by Holton (*6*). The procedure employed with Delafield's haematoxylin was as follows: The preparation was dried, then fixed in dilute Carnoy's solution for 2 to 5 minutes; flooded with water, which was removed with filter paper or cotton swab; dried over a very low flame; stained 3 to 30 minutes; washed by flooding with water; destained in acid alcohol; transferred to 70-percent alcohol and dehydrated through the higher alcohols; and then cleared and mounted in Canada balsam.

The nuclear behavior and distribution of the protoplasmic contents of the fused sporidia and binucleate hyphae in *Ustilago maydis* were found to differ in some respects from the descriptions for other members of the Ustilaginaceae. Several investigators (*1, 6, 8, 9, 11, 12, 13, 16*) agree essentially that after fusion the protoplasmic contents of the fused sporidia migrate into the fusion hypha and thereby initiate the binucleate phase. The contents then continue to move toward the apex as the hypha elongates. Sleumer (*16*), Rodenhiser (*13*), and Holton (*6*), investigating *U. zeae*, *Sphacelotheca sorghi* and *S. cruenta*, and *U. avenae* and *U. levis*, respectively, showed the formation of cross walls in the basal portions of old binucleate hyphae, although each of the cells thus formed, as well as the fused sporidia, apparently was empty. Stakman, Cassell, and Moore (*17*) found essentially the same situation in *Urocystis occulta*. However, they occasionally observed two-celled fused sporidia in which the nucleus was present in the nonfused cell.

In the present study, definite, well-defined nuclei were observed frequently in the fused sporidia and in the older basal portions of the binucleate hyphae, but their occurrence was more or less sporadic. Some cells both of sporidia and of hyphae apparently still contained the normal protoplasmic contents, whereas in others the contents were in various stages of disintegration and some cells appeared to be empty. The representative nuclear condition of the fused sporidia and older portions of the binucleate hyphae are illustrated in figure 2. In figure 2, *A*, the direct H-shaped type of fusion between two sporidia has just occurred. Both sporidia are two-celled. A single nucleus is evident in each of the fused cells and also in one of the nonfused cells. This last cell has produced a uninucleate hypha, which would make it possible for the one sporidium to fuse with two different sporidia or for a double fusion between two sporidia to occur.

It was possible to follow the entire binucleate hypha that had developed from the fused sporidia shown in figure 2, *D*. Representative portions of this hypha are shown in *E*, *F*, and *G*. The protoplasmic contents of the cells in *D* apparently were disintegrating, although scattered nuclei were clearly visible. In the fourth cell from the point of fusion (*E*, *a*) no nuclei were evident and only one each was observed in the fifth (*E*, *b*) and sixth (*E*, *c*) cells. The distribution of the cytoplasm in all three cells was rather irregular. In the middle portion of this hypha, the sixteenth cell (*F*, *b*) contained two clearly defined nuclei. Only one nucleus was visible in the seventeenth cell (*F*, *c*), and none was visible in either the fifteenth (*F*, *a*) or the eighteenth (*F*, *d*) cell. The cytoplasm, although not so uneven or patchy as in the older cells, was nevertheless in sharp contrast to the regular dis-

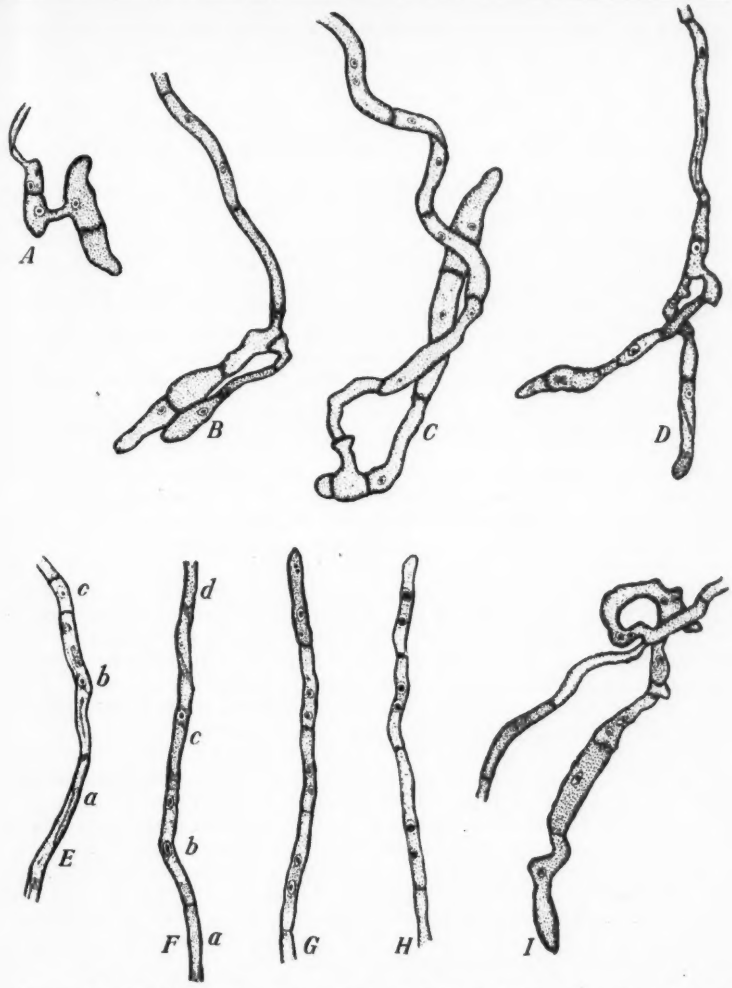

FIGURE 2.—Sporidial fusion and nuclear condition in *Ustilago maydis*. Approximately ×1,825. *A*, An early stage in the direct H-shaped type of sporidial fusion. Both sporidia are two-celled. The binucleate hypha has not yet developed. *B*, Sporidial fusion by means of short, uninucleate hyphae, or germ tubes, developed from ends of compatible sporidia. Paired nuclei are visible in the second cell of the binucleate hypha. *C*, Sporidial fusion in which one cell of one sporidium has functioned as the fusion cell. Paired nuclei are shown in three cells of the binucleate hypha. *D*, Fused sporidia. *E* to *G*, Representative portions of the binucleate hypha developed from the fused sporidia in *D*. The irregular cytoplasmic and nuclear condition commonly found in fused sporidia and older portions of binucleate hyphae is shown in *D*, *E*, and *F*. *G*, Paired nuclei in the tip cell of the hypha. *H*, Paired nuclei in the three apical cells of a representative binucleate hypha. *I*, Irregular and branched binucleate hypha. *A*, *B*, *C*, and *I*, stained with acid fuchsin; *D* to *G*, with Delafield's haematoxylin; *H*, with orseilline BB and aniline blue. (See text, p. 238, for explanations of *a*, *b*, *c*, and *d*.)

tribution of that in the younger cells near the tip of the hypha, as shown in *G*. There was also a decided difference in the staining reaction of the older and younger portions of the hypha. The older cells that contained one or more nuclei each, although staining more densely than those in which no nuclei were apparent, did not retain as much of the stain as did the rapidly growing cells at the end of the hypha.

Sleumer (*16*) distinguished three and sometimes four nuclei in the tips of binucleate hyphae in *Ustilago zeae* but did not show pairs of nuclei separated by cell walls. Allison (*1*), reporting on *U. levis* and *U. hordei* (Pers.) Lagerh., pictured one hypha containing six nuclei and another containing four, but no septa in either. Stakman, Cassell, and Moore (*17*) found that the binucleate hyphae of *Urocystis occulta* often contain two pairs of nuclei, frequently three nuclei, but rarely a single nucleus. They assume that the fourth and the second nucleus, respectively, may not have been visible or that one nucleus had not yet divided.

In the present study, paired nuclei, clearly delimited by septa, have been commonly observed in the end cells of binucleate hyphae. Paired nuclei were commonly found in the two end cells and occasionally in as many as five contiguous cells at the end of a binucleate hypha. Representative examples are illustrated in figure 2, *G* and *H*. In *G*, two small, darkly stained bodies are just visible in the tip of the apical cell. Whether these represent two daughter nuclei produced by division prior to cell-wall formation could not be definitely determined. The preceding five cells of this hypha (not illustrated) were well stained, but no pairs of nuclei could be definitely determined. The nucleoli shown in the three end cells of another binucleate hypha (*H*) appear to be slightly larger than those in other illustrations. This may have been due to the use of orseilline BB and aniline blue in place of Delafield's haematoxylin in the staining procedure.

Occasionally irregularly formed and branched binucleate hyphae (fig. 2, *I*) were observed. Sleumer (*16*) described a somewhat similar condition, which he termed "Wirkopulation." Inasmuch as the binucleate hyphae become branched and irregular in form within the host tissues, the occurrence of this condition in culture would seem to be more or less expected and not an exception in need of an explanation. One cell apparently contains two pairs of nuclei. The failure of a cell wall to form might have caused this condition; or possibly, as suggested by the shape of the cell, a new branch was beginning to develop into which the nuclei would migrate.

DISCUSSION

That the binucleate stage in the smut fungi is initiated in culture by the fusion of basidiospores (sporidia) has been demonstrated for a number of species in the Ustilaginaceae. However, the ability of the sporidia of *Ustilago maydis* to fuse in culture has not been clearly established previously. In the present study the fusion of compatible sporidia was obtained in culture. Its occurrence was found to depend largely on nutrition and temperature. Both factors influenced the time required for fusion to take place. In certain other smuts fusion occurs in a relatively short period of time. Holton (*6*) obtained fusion in *U. avenae* and *U. levis* after 35 minutes to 4 hours. Stakman,

Cassell, and Moore (*17*) observed fusion between the sporidia of *Urocystis occulta* as soon as the sporidia were fully formed. Rodenhiser (*13*) observed fusion in *Sphacelotheca sorghi* and *S. cruenta* after 10 hours. In the present study, however, fusion was first detected after 15 to 20 hours and was not readily observed until after 40 to 48 hours. The relatively long period of time and the extremely low concentration of nutrients, which apparently are necessary for sporidial fusion, may account for the negative results of many previous investigators.

From the present investigation of *Ustilago maydis* it could be deduced that the general nuclear behavior might be as follows. Simultaneously with, or immediately after, conjugation, the nucleus in each of the fusing gametes divides and a daughter nucleus migrates into the fusion tube or cell. Cell division is completed in the normal manner by means of cell-wall formation. Further growth of the binucleate hyphae is accomplished by normal cell division, i. e., nuclear division, migration of daughter nuclei, and cell-wall deposition. Each cell of the binucleate hyphae, therefore, normally contains one pair of nuclei.

The gradual aging and decline of the cells in the older portion of the hypha simultaneously with the growth of the apical region suggest that a disintegration of the protoplasmic contents of the older cells occurs at a rate depending upon the environmental conditions present in artificial culture. The extent to which this process is influenced by any particular set of environmental or nutritional conditions was not determined. The existence of physiological or biochemical differences between different portions of the hyphae is indicated by the differences in dye absorption and retention. Further studies might indicate a situation somewhat analogous to that described by Thomas (*18*) for several species of *Pythium*, in which he found that the fixation of dyes is determined by differences in cell-wall composition of young and that of old, mature hyphae. Whether the cells appear devoid of contents or contain nuclei either singly or in pairs would depend, therefore, upon the degree of protoplasmic aging and disintegration as well as upon the success of the staining technique.

If this interpretation is correct, the presence of empty fused sporidia and empty cells in old portions of binucleate hyphae or the appearance of cells with irregular numbers of nuclei might better be explained on the basis of physiological aging and disintegration of cell contents than of an irregular or unusual type of nuclear and cellular division. The viewpoint frequently expressed in the literature that the protoplasmic contents of fused sporidia pass into the fusion hypha and migrate toward the tip as growth occurs, leaving empty cells behind, was not found in this study to be applicable to *Ustilago maydis*.

SUMMARY

The production and fusion of sporidia in cultures of *Ustilago maydis* are described. The initiation of the binucleate stage by the fusion of compatible sporidia is described and illustrated. The fusion process was influenced markedly by the nutritive value of the culture media and by the incubation temperature.

Fused sporidia were sufficiently numerous to be readily detected after 20 hours at 24° C. in distilled water and after 15 to 20 hours at 20° to 24° in 1-percent malt-extract solution.

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The initiation of the binucleate phase followed no rigidly fixed method but resulted from the fusion of any two compatible haploid cells, either sporidial or hyphal. In no case were the binucleate hyphae observed to revert to sporidial production.

End cells of binucleate hyphae uniformly contained one pair of nuclei each. The protoplasmic contents of the older binucleate cells and of the fused sporidia appeared to be in various stages of disintegration. In some cases the cell contents were apparently normal, whereas in others they were either partly disintegrated or entirely lacking.

The rapidly growing apical cells of binucleate hyphae and young sporidia exhibited a pronounced affinity for the several dyes used, whereas older cells and old fused sporidia did not.

Since the results presented are based on a study of the fungus in culture they may or may not represent the situation as it exists in nature in the corn plant.

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UTILIZATION OF NITRATES BY THE COFFEE PLANT UNDER DIFFERENT SUNLIGHT INTENSITIES [1]

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INTRODUCTION

In the course of an investigation (*7*) [3] dealing with the levels of nitrogen, potassium, and carbohydrates in the leaves of coffee trees (*Coffea arabica* L.) grown at Kona, T. H., it was found that the leaves of young coffee trees (2 to 10 months old) accumulated relatively large amounts of nitrate nitrogen as compared with the leaves of other plants, and that the nitrate content fluctuated widely during the growing season. No adequate explanation for this seemingly unusual behavior was found. For this reason a study was made to determine the cause or causes which lead to the accumulation of nitrates in coffee leaves. After a consideration of the important external factors that influence nitrate metabolism, it appears that at Kona sunlight intensity might be the limiting factor in the process; hence, in this investigation the chief emphasis was placed on the effect of different degrees of sunlight intensity on the assimilation of nitrates by the coffee plant. In order to maintain strict control over the nutrient supply, the coffee plants were grown in water culture, This paper presents the results of the investigation.

REVIEW OF LITERATURE

A review of the factors that play a dominant role in the nitrate nutrition of green plants has been published by Nightingale (*21*).

The nitrate content of plants varies with different treatments and with the stage of growth (*1, 4, 6, 9, 35*). When the external supply of nitrate is plentiful many plants store large amounts in their roots, stems, or leaves without injurious effects (*1, 4, 6, 19, 35, 40*). Nitrates are known to accumulate in plants when carbohydrate synthesis is curtailed or when sunlight intensity is reduced (*11, 32, 33*), when temperature is low (*20, 23, 25*), and when potassium (*22, 26 34*) phosphorus (*8, 10, 34*), or manganese (*17, 39*) is deficient. Under conditions of high concentration of salts in the culture solution, the reduction and assimilation of nitrates within the plant become somewhat limited (*24*). Plants growing under low soil moisture conditions have also been found to accumulate nitrates (*12*).

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[3] Italic numbers in parentheses refer to Literature Cited, p. 256.

EXPERIMENTAL METHODS

The coffee seeds used in this study were obtained from a single tree at Kona, Hawaii. After the seedlings had grown for 3 weeks in a very dilute nutrient solution, two seedlings were transferred to each of a number of gallon jars containing the complete nutrient solution. The solutions were changed once every 3 weeks. The culture solutions were aerated moderately and continuously, and their reaction was maintained between pH 4.5 and pH 6.5. The composition of the complete nutrient solution was as follows: [4]

KNO_3—0.0012 M
$Ca(NO_3)_2$—0.0012 M
KH_2PO_4—0.00025 M
$MgSO_4.7H_2O$—0.0012 M
5 cc. of a 2-percent stock solution of ferric tartrate.
5 cc. from a liter of solution containing 0.25 gm. H_3BO_3, 0.5 gm. $MnCl_2.4H_2O$, 0.25 gm. $ZnSO_4$, 0.1 gm. $CuSO_4.5\ H_2O$.

On February 24, 1943, 44 plants were placed in the open, 24 plants were placed under moderate shade, and 24 plants under heavy shade. The moderate shade consisted of laths 2.5 cm. in width spaced 2.5 cm. apart, and the heavy shade consisted of 5.0-cm. laths spaced 2.5 cm. apart. After 3 weeks, the width of the laths of the heavy shade was increased to 7.5 cm., but the spacing was kept at 2.5 cm. Hereafter in this paper the moderate shade is referred to as one-half shade and the heavy shade as three-fourths shade.

Growth measurements and sunlight radiation records were made weekly, beginning on February 24. Sunlight intensity was measured by the decomposition of oxalic acid in 0.01 M uranyl sulfate solution. It has been found that reliable sunlight measurements can be obtained by the use of this method.[5]

TABLE 1.—*Outline of treatment of coffee plants in experiment begun Feb. 24, 1943*

Experiment No. and series	Number of plants	Treatment
1:		
A	8	Complete nutrient; no shade.
B	6	Complete nutrient; one-half shade.
C	6	Complete nutrient; three-fourths shade.
2:		
A [1]	8	3 weeks before sampling −N; no shade.
B [1]	6	3 weeks before sampling −N; one-half shade.
C [1]	6	3 weeks before sampling −N; three-fourths shade.
3:		
A [1]	8	3 weeks −N followed by 3 weeks +N; no shade.
B [1]	6	3 weeks −N followed by 3 weeks +N; one-half shade.
C [1]	6	3 weeks −N followed by 3 weeks +N; three-fourths shade.
4:		
A [2]	8	6 weeks before sampling −K; no shade.
B [2]	6	6 weeks before sampling −K; one-half shade.
C [2]	6	6 weeks before sampling −K; three-fourths shade.
5	6	6 weeks before sampling −P; no shade.
6 [3]	6	6 weeks before sampling +2 N; no shade.

[1] KNO_3 and $Ca(NO_3)_2$ were replaced by equimolar concentrations of KCl and $CaCl_2$.
[2] KNO_3 and KH_2PO_4 were replaced by equimolar concentrations of $NaNO_3$ and NaH_2PO_4.
[3] KH_2PO_4 was replaced by an equimolar concentration of KCl.

[4] Preliminary investigations showed that best growth was made in solutions of low osmotic pressure.

[5] From personal correspondence with H. W. Brodie of the Hawaiian Sugar Planters' Association. Honolulu, T. H. 1942.

On June 30, approximately 27 weeks after germination, the coffee plants were divided into six series and the treatments shown in table 1 were begun. After 6 weeks of these treatments, mature leaves from all plants were sampled. The fresh leaves were weighed, dried under strong draft in an electric oven at 80°–90° C., and ground to pass a 40-mesh screen.

In order to see whether similar results would be obtained during the cooler months of the year, the entire experiment was repeated with the same plants. On October 5 one plant was placed in each jar, and thereafter the solution was changed once every 2 weeks. On December 16, except for the plants in the nitrogen series, the plants were given the treatments mentioned previously. The plants in the nitrogen series were without nitrogen for 4 weeks. On January 27, 1944, leaves were sampled and treated in the same manner as before.

ANALYTICAL METHODS

Total nitrogen was determined by the Kjeldahl method after preliminary reduction with reduced iron powder (*27*).

Soluble nitrogen was extracted from the dried tissue with distilled water (*37*) and was determined as total nitrogen.

Insoluble nitrogen was determined by subtracting soluble nitrogen from total nitrogen.

Nitrate nitrogen was determined in an aliquot of the soluble nitrogen extract by reduction with Devarda's alloy.

Ammonia and amide nitrogen were analyzed in an aliquot of the soluble nitrogen extract by the method of Pucher et al. (*28*).

Alpha-amino nitrogen was determined in the residue from the ammonia determination by the Van Slyke method (*18*).

Peptide nitrogen was analyzed in an aliquot of the soluble nitrogen extract by the acid hydrolysis method of Vickery et al. (*36*).

Basic nitrogen was determined in an aliquot of the soluble nitrogen extract with phosphotungstic acid as the precipitant.

Soluble sugars were extracted with hot 80-percent alcohol, and after hydrolysis with invertase (*18*), were determined by the method of Quisumbing and Thomas (*3*).

Starch was determined in the residue from the alcohol extract (*18*).

Hemicellulose was determined in the residue from the starch determination (*18*).

Potassium was determined by the Na-colbaltinitrite method of Volk and Truog (*38*). The tissues were wet-ashed with a nitric-perchloric acid mixture.

Phosphorus and calcium were analyzed in the wet-ashed tissues by standard methods.

EXPERIMENTAL RESULTS

APPEARANCE OF PLANTS

The appearance of the coffee plants was greatly affected by the different light treatments. This can be seen in figure 1, where photographs are shown of typical plants from each light treatment.

Contrary to the results obtained by some others (*2, 29, 31*), who have investigated the effects of shading on the coffee plant, no ill effects from strong sunlight were manifested by the unshaded plants despite their gross structural differences from the shaded plants.

Some of the morphological differences arising from differences in shading were similar to those previously reported. Thus, shading increased the length of internodes (*2*, *29*, *30*) and size of leaves (*2*, *13*, *29*, *30*) but decreased the size of the root system (*14*) and the trunk. There were more leaves per plant in the unshaded series than in either of the shaded series. Plants grown under three-fourths shade appeared rather spindling and had the smallest number of leaves per plant. In most respects, the plants grown under one-half shade

FIGURE 1.—Coffee plants grown under three different light conditions. *A*, No shade; *B*, one-half shade; and *C*, three-fourths shade.

occupied an intermediate position between those grown without shade and those grown under three-fourths shade.

In all the minus-nitrogen series (table 1, experiments 2 and 3) the unshaded leaves were yellowish green after the period during which they were without nitrogen. In the case of the minus-potassium series (experiment 4) the leaves of the plants grown without shade developed symptoms of potassium deficiency. Necrotic areas appeared along the margins of mature leaves and enlarged as the deficiency progressed. In figure 2 are photographs of three leaves showing various stages of potassium deficiency. The plants grown without phosphorus (experiment 5) showed no visible signs of its absence.

RELATION BETWEEN GROWTH AND SUNLIGHT INTENSITY

In many coffee-producing countries the coffee plant is grown under shade because of the general belief that shading is essential for the best growth of coffee plants, especially those of the Arabian type. Yet, as far back as 1901, after a canvass of the subject, Cook (*5*) came to the conclusion that there is no basis for the belief that shade is necessary for the growth of the coffee plant.

The growth of the coffee plants and the sunlight intensity during the first experiment are graphically depicted in figure 3. Sunlight radiation is expressed as milliequivalents of oxalic acid decomposed. From the curves for cumulative sunlight, it can be seen that the one-half shade series received only 40 percent of the full sunlight, while the three-fourths shade series received about 20 percent growth and sunlight records obtained during the second experiment were similar to those of the first experiment. However, the weekly sunlight intensity record of the second experiment was about 75 percent of that of the first experiment.

During the first half of the first experiment the growth of the plants under the three light conditions appeared to be similar, but toward the end of the second half of the experiment the growth of the plants without shade was better than that of the shaded series (fig. 3). To determine whether the difference in growth among the three series

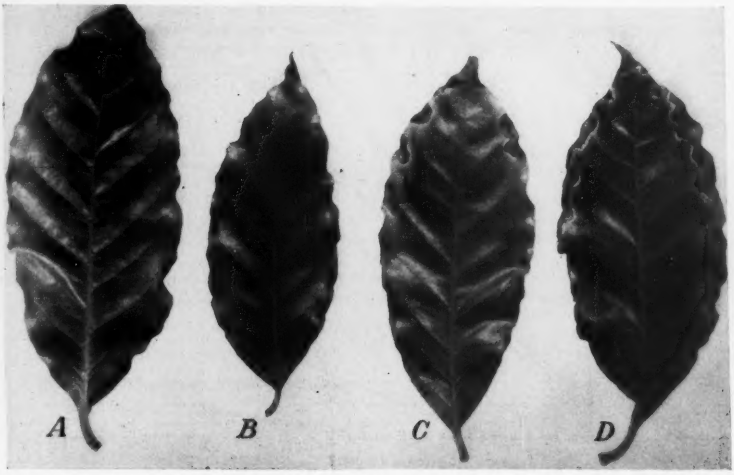

FIGURE 2.—*A*, Normal coffee leaf; *B-D*, coffee leaves showing progressively increasing severity of potassium deficiency.

were significant, the data were statistically examined by analysis of variance. In so doing, the growth period was divided into two parts—March 1 to May 3 and May 3 to June 28—and the increases in growth during the two periods were calculated. Table 2 contains the data showing the average increase in height during the two periods.

Statistical evaluation of the growth data showed that during the early stages of growth, the coffee plants grew better with half shade than with no shade (table 2), but during the latter half of the first experiment, the growth of the plants without shade was significantly better than that of the plants under one-half or three-fourths shade. The growth of the plants under one-half shade was also significantly better than that of the plants under three-fourths shade. When the growth data of the second experiment were evaluated, however, the results indicated that growth of the plants without shade was not

significantly better than that of the plants with one-half shade (table 2). Nevertheless, the growth of the unshaded plants and that of the one-half shade plants were significantly better than the growth of the three-fourths shade plants. These results and those of the first experiment indicate that the growth of the coffee plants was decreased by heavy shading.

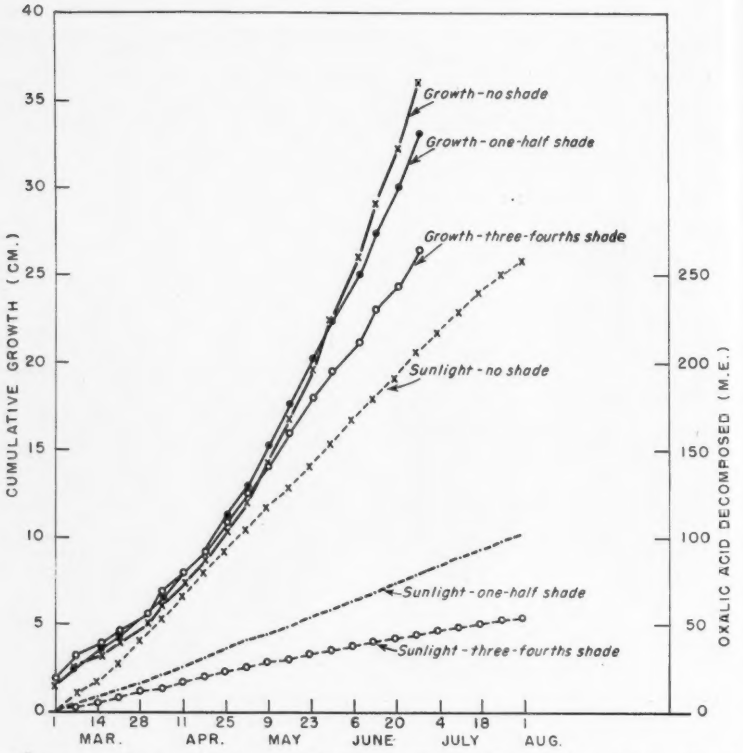

FIGURE 3.—Curves showing cumulative growth of coffee plants and intensity of sunlight during the growing period.

TABLE 2.—*Average increase in height, during 3 growth periods, of coffee plants receiving no shade, one-half shade, and three-fourths shade, 1943*

Series	Shade	Experiment 1		Experiment 2
		Mar. 1–May 3 [1]	May 3–June 28 [2]	Oct. 5–Dec. 13 [3]
		Cm.	*Cm.*	*Cm.*
X	None	10.4	24.1	16.5
Y	One-half	11.6	20.0	14.5
Z	Three-fourths	10.6	13.9	10.0

[1] Difference necessary for significance $P=0.01$—X–Y (or Z) ... 1.2 cm.; Y–Z ... 1.4 cm.
[2] Difference necessary for significance $P=0.01$—X–Y (or Z) ... 1.9 cm.; Y–Z ... 2.1 cm.
[3] Difference necessary for significance $P=0.01$—X–Y (or Z) ... 3.8 cm.; Y–Z ... 4.4 cm.

The results obtained from the trunk measurements of the coffee plants seem to confirm the results obtained from the growth measurements. From table 3 it can be seen that unshaded plants were significantly larger in circumference than the plants of either shaded series. The circumferences of the one-half shade plants were also larger than those of the three-fourths shade plants.

CHEMICAL ANALYSES OF COFFEE LEAVES

The results of the chemical analyses of the coffee leaves are recorded in tables 4 to 7.

Dry-matter content.—Although the dry-matter content of the leaves differed somewhat in the different light series, dry matter was decreased by shading in all series (table 4). Severe potassium deficiency resulted in an increase in dry-matter content (experiment 8), probably because of the necrotic tissues.

TABLE 3.—*Average circumference of trunks of coffee plants receiving no shade, one-half shade, and three-fourths shade*

Series	Shade	Circumference [1]
		Cm.
X	None	4.5
Y	One-half	3.5
Z	Three-fourths	2.8

[1] Difference necessary for significance $P=0.01$—X–Y (or Z) . . . 0.2 cm.; Y–Z . . . 0.2 cm.

TABLE 4.—*Moisture content of leaves from coffee plants receiving no shade, one-half shade, and three-fourths shade, with and without complete nutrient*

[On green-weight basis]

Experiment No. and series	Shade	Nutrient	Moisture	Dry matter
1:			*Percent*	*Percent*
A	None	Complete	72.83	27.17
B	One-half	do	74.35	25.65
C	Three-fourths	do	75.35	24.65
2:				
A	None	−N, 3 weeks	73.39	26.61
B	One-half	do	74.50	25.50
C	Three-fourths	do	75.00	25.00
3:				
A	None	−N, 3 weeks; +N, 3 weeks	72.58	27.42
B	One-half	do	73.30	26.70
C	Three-fourths	do	74.10	25.90
4:				
A	None	−K, 6 weeks	71.00	29.00
B	One-half	do	72.84	27.16
C	Three-fourths	do	74.00	26.00
5	None	−P, 6 weeks	72.57	27.43
6	do	+2 N, 6 weeks	71.42	28.58
7 [1]	do	Complete	67.51	32.49
8 [1]	do	−K	65.23	34.77
9 [1]	do	−P	68.98	31.02

[1] Plants used in preliminary experiment. They were given treatment indicated until definite deficiency symptoms appeared.

Total nitrogen.—Total nitrogen increased with shading (table 5, experiment 1). Withholding nitrogen from the plants resulted in a decrease in the total nitrogen content and reduced the differences in nitrogen content existing among the plants under different light treat-

ments (experiment 2). These results showed that a good proportion of the nitrogen in the shaded plants was storage nitrogen. As expected, increasing the external nitrogen supply increased the total nitrogen content of the leaves.

TABLE 5.—*Nitrogen in leaves of coffee plants receiving no shade, one-half shade, and three-fourths shade, with and without complete nutrient*

[On dry-weight basis]

Experiment No. and series	Shade	Nutrient	Total N	Soluble N	Insoluble N	Nitrate N	Basic N
1:			*Percent*	*Percent*	*Percent*	*Percent*	*Percent*
A	None	Complete	2.72	0.772	1.95	0.027	0.514
B	One-half	do	3.18	.895	2.28	.123	.520
C	Three-fourths	do	3.44	.971	2.47	.230	.494
2:							
A	None	−N, 3 weeks	2.51	.705	1.80	.019	.499
B	One-half	do	2.56	.679	1.88	.024	.480
C	Three-fourths	do	2.66	.680	1.98	.057	.472
3:							
A	None	−N, 3 weeks, +N, 3 weeks.	2.44	.663	1.78	.015	.464
B	One-half	do	2.59	.621	1.97	.045	.444
C	Three-fourths	do	3.04	.777	2.26	.105	.445
4:							
A	None	−K, 6 weeks	2.78	.821	1.96	.021	.502
B	One-half	do	3.02	.804	2.22	.053	.495
C	Three-fourths	do	3.32	.949	2.37	.078	.476
5	None	−P, 6 weeks	2.98	.830	2.15	.063	.525
6	do	+2 N, 6 weeks	3.47	1.193	2.28	.143	.623
7 [1]	do	Complete	2.70	.715	1.98	.031	.483
8 [1]	do	−K	2.72	.878	1.84	.035	.568
9 [1]	do	−P	3.77	1.921	1.85	.214	.773

[1] Plants used in preliminary experiment. They were given treatment indicated until definite deficiency symptoms appeared.

Severe phosphorus deficiency (table 5, experiment 9) resulted in an abnormal accumulation of nitrogen. The figures for total and soluble nitrogen showed that this increase was due to an abnormal increase in soluble nitrogen.

Potassium deficiency did not seem to affect the total nitrogen content of the leaves.

Soluble nitrogen.—With an ample supply of nitrogen, soluble nitrogen increased with shading (table 5, experiment 1). The increase was largely due to the accumulation of nitrates. When nitrogen was withheld from the plants (experiment 2) there was a decrease in soluble nitrogen. During the second experiment the leaves of the unshaded plants tended to have less soluble nitrogen than those of the shaded plants.

Lack of potassium increased the soluble nitrogen content of the leaves to some extent, owing largely to an increase in the basic nitrogen fraction.

A severe lack of phosphorus (experiment 9) resulted in an abnormal increase in soluble nitrogen, chiefly due to increases in the amounts of nitrate and peptide nitrogen. Basic nitrogen and alpha-amino nitrogen were also accumulated by the phosphorus-starved plants.

Insoluble nitrogen.—Insoluble nitrogen increased with shading and decreased when the external nitrogen supply was cut off (experiments 1 and 2, table 5). Hopkins (*15*) and Kraybill (*16*) have reported similar results for other plants.

Nitrate nitrogen.—The amount of nitrate nitrogen in the leaves varied widely in the first experiment, ranging from a few thousandths

of 1 percent to over two-tenths of 1 percent (table 5). In the second experiment there was less nitrate nitrogen in the leaves, probably because they were more mature.

Although sunlight is known to influence the assimilation of nitrate by green plants (*21*), the low percentages of nitrate nitrogen in the leaves of plants grown without shade point to the relative unimportance of sunlight in the assimilation of nitrate nitrogen by coffee leaves. Nitrate nitrogen increased with increase in shading as well as with increase in the external supply. When there was no nitrate in the culture solution, the nitrate stored in the leaves was quickly used up by the plant (experiment 2). On the other hand, when it was again supplied to the plant, the excess was again stored by the plant (experiment 3). These results indicate that when there is an excess of nitrate the accumulation of nitrate by coffee leaves under adequate nutritional conditions is a normal phenomenon; and the degree of accumulation is largely determined by the amount of solar energy available to the plants.

Phosphorus starvation resulted in an abnormal accumulation of nitrates. Other workers (*8, 10, 34*) have reported similar results with other plants. Eckerson (*10*) attributed the accumulation of nitrate to the role played by phosphorus in reducase synthesis.

Ammonia and amide nitrogen.—From the analytical results, it appears that ammonia nitrogen and amide nitrogen were not accumulated to any large extent by the coffee leaves under the conditions of this experiment. The figures ranged from less than 1 p. p. m. to 13 p. p. m. The trend of the figures indicates that both types of nitrogen are found in larger amounts in shaded leaves. Both forms seemed to accumulate when the nitrogen supply was greater than the utilization. The lack of phosphorus resulted in increases in ammonia nitrogen and amide nitrogen.

Basic nitrogen.—The high percentage of basic nitrogen (table 5) in the leaves of coffee plants indicates that this nitrogenous fraction cannot be overlooked in a consideration of the soluble nitrogenous constituents of coffee leaves. Under normal conditions it often comprises more than half of the total soluble nitrogen fraction. Cutting off the external supply of nitrogen resulted in a small decrease in its amount. Severe potassium deficiency caused some accumulation of basic nitrogen at the expense of insoluble nitrogen. Lack of phosphorus also resulted in accumulation of basic nitrogen.

Alpha-amino nitrogen.—In the first experiment the alpha-amino nitrogen showed a tendency to increase with shading. In the second experiment, however, this trend was not observed. The amount of alpha-amino nitrogen ranged from 0.03 percent to 0.11 percent. Lack of potassium did not seem to affect the concentration of alpha-amino nitrogen, but the absence of phosphorus (experiment 9) resulted in a large increase in the level of this constituent.

Peptide nitrogen.—The behavior of the peptide nitrogen fraction was somewhat similar to that of alpha-amino nitrogen. The peptide nitrogen in the coffee leaves ranged from 0.01 percent to 0.21 percent. Phosphorus deficiency resulted in an accumulation of this constituent.

Soluble sugars.—In the summer experiment the soluble sugar content showed a decided decrease with increase in shading (table 6, experiment 1), but in the winter experiment the trend was less evident. There was some accumulation of soluble sugars when potassium was

omitted from the culture solution for 6 weeks (experiment 4), but under conditions of severe deficiency of potassium (experiment 8), soluble sugars did not accumulate in the leaves. Nightingale (*26*) also found that at the outset of potassium deficiency, carbohydrates accumulate; and, with further lack of potassium, they decrease.

Starch.—The starch content of the leaves showed a very marked decrease with increase in shading (table 6). The starch content of the leaves in experiment 2 was much higher than that of experiment 1; nevertheless, it also showed a strong tendency to decrease with shading.

Hemicellulose.—In experiments 1 to 4 the hemicellulose fraction showed a tendency to increase with increasing shade (table 6).

Potassium.—In all light treatments (table 7, experiments 1 to 4) the potassium content of the leaves increased with an increase in shading. This is believed to be the result of luxury consumption. When the percentage of potassium in leaves dropped below about 2 percent, necrotic areas appeared along the leaf margin.

Calcium.—The calcium content of the leaves in both experiments 1 and 2 increased with shading (table 7).

TABLE 6.—*Carbohydrates in leaves of coffee plants receiving no shade, one-half shade, and three-fourths shade, with and without complete nutrient*

[On dry-weight basis as dextrose]

Experiment No. and series	Shade	Nutrient	Soluble sugars	Starch	Hemicellulose
1:			*Percent*	*Percent*	*Percent*
A	None	Complete	6.23	3.71	7.38
B	One-half	do	5.83	.32	7.65
C	Three-fourths	do	5.05	.16	8.30
2:					
A	None	−N, 3 weeks	6.65	4.60	6.98
B	One-half	do	6.42	2.88	8.35
C	Three-fourths	do	5.48	1.28	8.80
3:					
A	None	−N, 3 weeks; +N, 3 weeks	5.60	2.18	7.65
B	One-half	do	5.95	1.46	7.80
C	Three-fourths	do	5.42	.10	8.48
4:					
A	None	−K, 6 weeks	8.55	4.02	7.35
B	One-half	do	8.02	.16	8.20
C	Three-fourths	do	6.10	.16	8.00
5	None	−P, 6 weeks	6.42	1.24	6.90
6	do	+2N, 6 weeks	5.22	.16	6.88
7 [1]	do	Complete	5.48	.34	6.88
8 [1]	do	−K	5.52	.16	7.00
9 [1]	do	−P	4.25	.16	6.98

[1] Plants used in preliminary experiment. They were given treatment indicated until definite deficiency symptoms appeared.

Phosphorus.—The results of experiments 1 and 2 (table 7) indicated that the phosphorus content of the coffee leaves was slightly reduced by the light treatment.

The behavior of the phosphorus-deficient plants, as revealed by the results of the chemical analysis for the various nitrogeneous fractions, showed that lack of phosphorus caused a very serious break-down in the normal process of nitrogen metabolism. The break-down seemed to be associated with the anabolic process of nitrogen metabolism.

TABLE 7.—*Ash constituents in leaves of coffee plants receiving no shade, one-half shade, and three-fourths shade, with and without complete nutrient*

[On dry-weight basis]

Experiment No. and series	Shade	Nutrient	Potassium	Calcium	Phosphorus
			Percent	*Percent*	*Percent*
1:					
A	None	Complete	2.89	1.46	0.20
B	One-half	do	3.40	1.60	.18
C	Three-fourths	do	4.06	1.66	.18
2:					
A	None	−N, 3 weeks	2.84	1.40	.20
B	One-half	do	3.10	1.53	.20
C	Three-fourths	do	3.52	1.58	.19
3:					
A	None	−N, 3 weeks; +N, 3 weeks	3.06	1.40	.20
B	One-half	do	3.58	1.56	.20
C	Three-fourths	do	4.06	1.58	.19
4:					
A	None	−K, 6 weeks	1.62	1.50	.18
B	One-half	do	1.82	1.58	.18
C	Three-fourths	do	2.49	1.80	.21
5	None	−P, 6 weeks	3.20	1.47	.10
6	do	+2N, 6 weeks	3.46	1.72	.20
7 [1]	do	Complete	2.74	1.33	.15
8 [1]	do	−K	.76	1.52	.16
9 [1]	do	−P	2.84	1.23	.06

[1] Plants used in preliminary experiment. They were given treatment indicated until definite deficiency symptoms appeared.

SUMMARY AND CONCLUSIONS

Coffee plants (*Coffea arabica* L.) were grown in water culture under three different sunlight intensities. The first study was conducted during the summer, the second during the winter. On the whole, the results obtained during the two seasons were about the same.

Under the conditions of this experiment, with height and trunk measurements used as indices, the coffee plants were found to grow better without shade than with heavy shade.

Plants grown without shade and under one-half shade appeared much hardier than those grown under three-fourths shade. The number of leaves per plant decreased with increase in shading, while the leaf size increased with shading. Unshaded plants had larger trunks and larger root systems than the shaded plants.

Chemical analyses of the leaves showed that increases in shading resulted in increases in total nitrogen, soluble nitrogen, insoluble nitrogen, ammonia nitrogen, amide nitrogen, nitrate nitrogen, alpha-amino nitrogen, peptide nitrogen, and hemicellulose; and resulted in decreases in dry matter, soluble sugars, and starch. Potassium and calcium tended to increase with shading. During the cooler period, phosphorus also tended to increase with shading.

Severe potassium deficiency resulted in an increase in the soluble nitrogen content. The increase was due principally to an increase in the basic nitrogen fraction. At the outset of potassium deficiency, soluble sugars accumulated but later fell back to normal values.

Severe phosphorus deficiency resulted in abnormal increases in total nitrogen, ammonia nitrogen, amide nitrogen, nitrate nitrogen, basic nitrogen, alpha-amino nitrogen, and peptide nitrogen. These results pointed to a serious break-down in the nitrogen metabolism of phosphorus-starved plants. The break-down seemed to be associated

with the anabolic process. Of the soluble nitrogeneous fractions, nitrate nitrogen showed the greatest relative increase.

It is concluded that under suitable nutritional conditions, the accumulation of nitrates in the leaves of the coffee plant is a normal process, and that the amount stored at a certain period is determined largely by the amount of solar energy available to the plant at that time. If the supply of nitrate nitrogen is larger than the demand, the excess is stored by the plant.

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RELATION OF COLOR-INHIBITING FACTOR TO SMUDGE RESISTANCE IN ONION [1]

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INTRODUCTION

The greater resistance of colored bulbs of onion (*Allium cepa* L.) than of white ones to the attack of the smudge organism (*Colletotrichum circinans* (Berk.) Vogl.) has long been known. The factors responsible for resistance have been given considerable study in recent years, chiefly with the object of developing resistant white varieties. The purpose of the study presented here was to determine the effect of different color genotypes upon resistance.

REVIEW OF LITERATURE

Berkeley (*2*),[4] who first described onion smudge, observed that it was common on white bulbs but that colored ones were highly resistant. In 1921 Walker (*10*) gave an extensive description of the disease and its relation to environmental factors. In a series of studies Walker and associates (*11*, *12*, *13*, *14*) showed that a water-soluble substance extracted from pigmented scales was toxic to the smudge organism and that this substance was not found in unpigmented scales. They showed also that the volatile substances present in onions were toxic, killing ungerminated spores and checking the growth of the mycelium. Later Walker and associates (*1*, *6*, *7*, *15*) demonstrated that one of the toxic substances present in pigmented scales was protocatechuic acid. Link and Walker (*8*) determined further that catechol, which was present in pigmented scales and absent from white scales, was even more toxic than protocatechuic acid to the onion smudge organism. Ingersoll, Vollrath, Scott, and Lindegren (*4*) found that the volatile substances from freshly crushed onion and garlic contained unsaturated aldehydes, which they suspected to be either allyl aldehyde (acrolein) or crotonaldehyde, both of which were shown to have high bactericidal activity.

The genetic factors responsible for pigmentation in the onion were first described by Rieman (*9*), who demonstrated the occurrence of two kinds of factors for white, one a dominant inhibitor and the other

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[4] Italic numbers in parentheses refer to Literature Cited, p. 264.

a recessive white factor. Later studies by Clarke, Jones, and Little (*3*) confirmed these results but showed that the factors differentiating red and yellow pigments were not allelomorphic with the recessive white factor, as postulated by Rieman (*9*), but were independent of it.

The dominant allele *C* is necessary for color development. A variety of onion homozygous for red has the genotype *iiCCRR*, for yellow *iiCCrr*, and for recessive white *iiccRR*, *iiccRr*, or *iiccrr*. The *I* gene is incompletely dominant in the heterozygous condition, but all bulbs homozygous for *II* are white (*3*, *9*).

MATERIALS AND METHODS

Several F_1 plants from each of six different crosses between white and yellow varieties were grown in the greenhouse at Beltsville, Md. The flowers were selfed, and the resulting seed was sent to Wisconsin for growing and testing. In addition, F_2 plants from a cross between Crystal Wax and Italian Red were selfed, and this seed, as well as that from the selfed white and colored parents, was sent to Wisconsin. At first it was thought that the Crystal Wax plant carried the recessive white factor *c* and the recessive allele of the dominant white factor *I*. The F_3 seed was, therefore, included for comparing the recessive white with the dominant white plants from other populations. However, it was discovered later that the Crystal Wax parent carried the dominant white factor and had the genetic constitution *IICC*, although most plants of this variety have the constitution *iicc*. One of the crosses from which F_2 populations were grown had as the white parent a strain of Southport White Globe. The F_2 populations gave a segregation of 13 white and cream to 3 colored. It was therefore concluded that the white parent had the genetic constitution *IIcc*. Since the white bulbs appearing in such populations consist of five genotypes phenotypically indistinguishable, it was decided to omit all these populations from the analysis. In the remaining five crosses the variety White Portugal was used as the white parent. Most of these plants had the constitution *IICC* and gave ratios of 1 white to 2 cream to 1 colored in F_2 populations from crosses with colored (*iiCC*) plants. As one of the White Portugal lines, 3–213, had the genotype *IIcc*, all populations involving this parent were excluded from the analysis.

The colored parents involved in the crosses were Stockton G36, Italian Red, Early Grano, Stockton Yellow Flat, and Yellow Globe Danvers. The last-named variety is very pungent, whereas the other four are mild types.

Seed was sown in flats in the greenhouse in early March. Plants were transplanted outdoors in early May. Inasmuch as it was not possible to replicate each lot, plots of susceptible tester stocks of Southport White Globe and White Sweet Spanish varieties were distributed at random throughout the plots. The area in which the plots were located had also grown onions the 2 years immediately preceding. All these onion crops were inoculated with the smudge organism. Gross cultures of *Colletotrichum circinans* were grown on aerated Czapek's solution and distributed over the lower parts of the plants when the bulbs were about half-grown. Overhead irrigation

was applied during the succeeding night to provide favorable conditions for infection.

In late August when the tops were mature the bulbs were removed from the soil and placed in shallow crates in a protected outdoor location to cure. Curing was allowed to proceed in accordance with the usual commercial practice until mid-November, during which period the disease continued to develop and advance from the dry outer scales to the fleshy ones (*10*).

Disease analysis, made during the week of November 15, consisted first of dividing the bulbs into three color classes: White, cream, and yellow or red. The bulbs in each color class were then divided into four disease classes as follows:

(0) No evidence of smudge.
(1) Slight evidence of smudge, usually on the outermost scale only.
(2) Moderate amount of smudge.
(3) Large amount of smudge, with invasion of one or more fleshy scales.

The number of bulbs in each disease class was multiplied by the class number, and the sum (S) of the products was obtained. The index was then calculated by the following formula:

$$\frac{S \times 100}{3 \times \text{total population}} = \text{disease index.}$$

The uniformity of infection throughout the disease plot is shown by the disease indexes for the tester stocks, Southport White Globe and White Sweet Spanish. When distributed at random throughout the test plot, plantings of Southport White Globe gave index readings of 100.0, 95.4, 96.2, and 97.4; and White Sweet Spanish, 82.7, 77.4, 74.8, 61.3, and 92.2.

The analysis of variance was employed to obtain comparisons between the three genotypes *II* (white), *Ii* (cream), and *ii* (colored) and also between the different crosses. Only populations that showed a satisfactory fit to a 1:2:1 ratio were included in the analysis. As pointed out by Clarke, Jones, and Little (*3*), it is easy to distinguish white from cream bulbs in some populations, whereas in others the two classes cannot be separated with certainty. No doubt the populations that showed a poor fit to the expected 1:2:1 ratio contained many bulbs which had been incorrectly classified as to genotype. It was felt that the mean index for each group of bulbs within the populations was the most reliable measure of reaction to smudge; and, in order to avoid giving undue weight to occasional escapes or errors in classification, only those populations which had at least five plants in the smallest class were included in the analysis.

RESULTS

In calculating the variation due to genotype with respect to the *I* gene, it was found that in all six crosses there was a significant difference in susceptibility between genotypes, the F value always being several times that required for the 1-percent level of significance. However, this is to be expected on the basis of repeated observations by many workers that white bulbs are more susceptible to smudge than colored ones. The question that has not been answered satisfactorily heretofore is whether bulbs heterozygous for the *I* gene are

more resistant to smudge than the homozygous white ones. In all six of the crosses the mean index of the *Ii* bulbs showed that they were more resistant to smudge than the *II* bulbs. In four of these crosses the differences were highly significant. In the other two the differences were not significant because the number of populations was so small that large differences were required. In all six crosses the differences between the indexes of the colored bulbs (*ii*) and of the cream bulbs (*Ii*) were highly significant. In all but one cross, White Portugal × Yellow Globe Danvers, the mean index of the heterozygous cream bulbs was slightly nearer the index of the white bulbs than that of the colored bulbs. When all crosses were considered, the mean index for all bulbs was 40.30 and that of the cream bulbs was 41.64. This small difference was not significant; so it can be concluded that from the standpoint of smudge susceptibility the *I* gene is scarcely, if at all, dominant over the *i* gene and that the heterozygous bulbs *Ii* are approximately intermediate between the two homozygous types in susceptibility.

The combined analysis of all the crosses (table 1) showed a difference due to genotypes and a highly significant difference between crosses, even though they all contained the same three genotypes as to color. It is evident, therefore, that factors besides color affect the susceptibility of the bulbs to smudge. The differences in susceptibility to smudge between crosses are shown in table 2.

TABLE 1.—*Summary of data on smudge resistance in segregating progenies from 6 onion crosses*

EXPERIMENTAL DATA

Cross	Populations	Disease indexes			
		II	*Ii*	*ii*	Mean
White Portugal × Stockton G36	4	88.42	69.88	21.80	60.03
Crystal Wax × Italian Red	5	77.94	58.94	16.52	51.13
White Portugal × Early Grano	21	72.71	51.07	23.49	49.09
White Portugal × Italian Red	8	78.12	51.29	3.86	44.42
White Portugal × Stockton Yellow Flat	4	72.32	43.62	5.88	40.61
White Portugal × Yellow Globe Danvers	21	46.01	18.66	5.98	23.55
Mean [1]		65.89	41.64	13.38	40.30

ANALYSIS OF VARIANCE

Source of variation	Degrees of freedom	Mean square	*F* values		
			Calculated	Required	
				5-percent level	1-percent level
Genotypes	2	43,505.90	310.22	3.08	4.80
Crosses	5	5,877.58	41.91	2.29	3.18
Populations (within crosses)	57	410.44	2.93	1.45	1.68
Genotypes × crosses	10	699.20	4.99	1.91	2.49
Error	114	140.24			

[1] Difference required for significance between genotype means at the 1-percent level=5.61.

TABLE 2.—*Differences in susceptibility to onion smudge between crosses*

[Basic data in table 1]

Cross	White Portugal × Stockton G36	Crystal Wax × Italian Red	White Portugal × Early Grano	White Portugal × Italian Red	White Portugal × Stockton Yellow Flat	White Portugal × Yellow Globe Danvers
White Portugal × Stockton G36		8.90	10.94*	15.61**	19.42**	36.48**
Crystal Wax × Italian Red	8.90		2.04	6.71	10.52*	27.58**
White Portugal × Early Grano	10.94**	2.04		4.67	8.48*	25.54**
White Portugal × Italian Red	15.61**	6.71	4.67		3.81	20.87**
White Portugal × Stockton Yellow Flat	19.42**	10.52*	8.48*	3.81		17.06**
White Portugal × Yellow Globe Danvers	36.48**	27.58**	25.54**	20.87**	17.06**	

*Significant; **highly significant.

DISCUSSION

The fact that heterozygous cream bulbs are approximately intermediate in resistance between the pure white and the colored bulbs is important from the standpoint of breeding. It would be highly desirable to obtain varieties of white onions resistant to smudge, but if resistance is inevitably associated with the presence of pigment it would be difficult, if not impossible, to attain such an objective. However, cream bulbs with the genetic constitution *Ii*, though containing some pigment, might be as acceptable as pure white bulbs for certain purposes. The increase in resistance to smudge would probably more than compensate for the sacrifice in color purity. Although it would be impossible to obtain true breeding strains with the constitution *Ii*, the production of hybrid onions with this constitution would be very simple. This could be accomplished through the use of male-sterile strains as described by Jones and Clarke (*5*).

The effect of the recessive white gene *c* upon resistance to smudge has not been studied and will be the subject of further investigations. A difference between the susceptibility of red and yellow bulbs resulting from the action of the *Rr* pair of alleles has never been demonstrated and should be investigated further. Differences in susceptibility between crosses involving red and yellow parents did exist in the data presented here, but such differences are probably to be ascribed to factors other than color. It is noteworthy that the highest and lowest mean indexes of susceptibility were found in crosses between white and yellow varieties.

SUMMARY

The most important factor in resistance to onion smudge is the presence of pigment in the outer scales of the bulbs. White bulbs homozygous for the dominant color inhibitor *I* are highly susceptible to the disease. Colored bulbs with the genetic constitution *ii* are highly resistant. Cream bulbs with the constitution *Ii* are approximately intermediate in resistance between the pure white and the colored ones.

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